***Original Article***

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# Surrounding gas composition affects the calling song development in the two-spotted cricket (*Gryllus bimaculatus*)

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**SUMMARY** Male crickets emit acoustic signals (*i.e.*, songs) by chirping using their forewings. Although the mechanisms and adaptive functions of these songs are well studied, knowledge about how songs develop within a generation is relatively scarce. Our previous work demonstrated a stable peak frequency at 5.7 kHz in the calling songs recorded from mature adult male crickets (*Gryllus bimaculatus*). In the present study, we monitored changes in the frequency component over time from the sexual maturity stage (early adult stage). We recorded 300 calling songs from a pool of 122 adults. The peak frequency distribution was lower and unstable (*i.e.*, greater coefficient of variance) in the early adult stage. The mean peak frequency was 4.9 kHz on day 3, but gradually converged to 5.8 kHz over the 2-week adult stage. Immature adult males (emitting immature songs) produced an appropriately tuned song with a peak frequency of 5.8 kHz in an environment of 80% helium and 20% oxygen. These results suggest that the frequency component of the calling song is acquired during the early to mid-adult stage, and may be related to sexual maturation in males. Findings from the helium substitution experiment revealed that physical resistance from surrounding gas molecules negatively affect the stability of male singing, and that muscle development and forewing hardening may contribute to the maturation of singing, suggesting that females may adaptively select sexually mature males based on song traits.

***Keywords*** Cricket, *Gryllus*, calling, bioacoustics, development

## 1. Introduction

Acoustic signals are widely used for communication between members of the animal kingdom, including primates (*1,2*) , birds (*3-5*) , frogs (*6*) , and arthropods (*7-9*). In human clinical practice, abnormal acoustic signals (voice) are often found in patients with sicknesses. Such vocal abnormalities are due to physical disturbances in the vocal organs or functional disturbances in the neural circuits involved in vocalization. Furthermore, the tone of a person's speech reflects to some extent their mental health, and understanding the function, mechanism, and development of vocalization is important in achieving wellness for people.

The physical parameters that characterize acoustic signals are frequency (corresponding to pitch), amplitude (corresponding to volume), or more complex syllable patterns (*10-12*). The frequency spectrum is specific to each species (*13,14*), and closely related species produce songs with relatively different frequencies (*15*). The frequency components are continuously distributed to form a frequency spectrum, which is associated with premating isolation (*16,17*) such that females are likely to be attracted only to mating songs of the appropriate frequency, while ignoring other songs. In the two-spotted cricket *Gryllus bimaculatus*, we reported that the dominant frequency of song calling is tightly controlled and not affected by the size of the male's body or the size of the resonator (mirror and harp region of the forewing) (*9*). Based on the idea of directional hearing, this trait would be adaptive for males because songs with an inappropriate frequency may not transfer the information of the song emitter's (*i.e.*, male's) position to conspecific females. This function is especially critical in mating contexts, where females being attracted by male calling songs seek conspecific males on the basis of calling songs as a navigator. Another example of the biologic significance of frequency components is directional hearing. In crickets, a series of mechanistic studies demonstrated that the auditory system has directional hearing and can identify the direction of a sound source (*18-20*). Several physical models are proposed for directional hearing, including amplitude, time, and phase differences between the left and right ears. Studies have demonstrated that such directionality is selectively observed near the dominant (main peak) frequency of the calling songs of crickets (*18,19*).

The detailed mechanisms by which crickets produce their specific chirping patterns are well documented in a number of studies. Theoretically, the harmonic frequency of a membrane instrument (*e.g.*, tympani) is affected by the membrane density, tension, and membrane diameter (size) (*21*). In a previous study, we found that the size (membrane area) of the 2 major sound sources (harp and mirror region of the forewings) of *G. bimaculatus* varies with the body size (*9*), suggesting that the constant frequency of cricket song is determined by other factors, such as membrane tension and density. Nonetheless, another approach to understanding the behavior (*i.e.*, the tight regulation of song frequency) is to calculate how it develops over the course of a generation (cf., Tinbergen's 4 questions). There are no reports on how the tightly regulated song frequency is acquired during the development in *G. bimaculatus*.

In the present study, we examined whether the characteristic song pattern of mature *G. bimaculatus* calling songs is age-dependent. Elucidation of the relationship between age and the song pattern may provide meaningful insights and new hypotheses for cricket ethology. By recording the mating songs of a large number of crickets, we found that adult male crickets gradually begin to emit mating songs from the day 3 after reaching adulthood, but it takes about 1-2 weeks for these mating songs to reach maximum activity. To provide a physical explanation for the song maturation, we conducted experiments in which the air was replaced by 80% helium (which has a lighter average molecular weight than air) and the songs were recorded. Although the neuromuscular development of the forewing control is likely to contribute to the song maturation, it is virtually impossible to directly manipulate these factors in *G. bimaculatus*. Hence, instead, we manipulated the molecular weight of the environmental gases that resist the movement of the forewings.

## 2. Materials and Methods

### 2.1. Crickets

Crickets were purchased from Tsukiyono Farm (Gunma, Japan) and reared at 28ºC on a 12-h light and 12-h dark cycle as previously reported (*22,23*). Food and water were provided *ad libitum.* Crickets were separated in plastic containers (1 cricket per container) at the last nymphal stage and recorded on the day of the last molt (designated as the first day of the adult stage). All crickets were maintained at 28°C. Three batches of crickets were purchased for this study. For quality control purposes, we checked the survival rates of the 3 batches and found no difference in survival rates among batches (Supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=120*).

### 2.2. Cricket song recordings

Cricket songs were recorded using a microphone (F-112, Sony Inc., Tokyo, Japan) connected to a linear pulse-code modulation (PCM) recorder (PCM-D100, Sony Inc.). The sampling rate was 48,000 Hz and the data were saved as uncompressed 16-bit waveform files. Crickets that emerged as adults on different days were recorded in a quiet room under white fluorescent light from January 23 to March 12, 2017. A sound recorder was placed in front of several cups containing crickets to record the crickets' songs. The experimenter sat in the room and waited for the crickets to chirp. When a cricket chirped, the experimenter recorded the cricket's identification (ID) number and the date and time it was recorded. If more than one cricket chirped at the same time, the experimenter rattled the container to silence them and then waited for each to chirp independently. Crickets whose songs were recorded for the day were transferred to an incubator (so that they would not be recorded again later that day). Recordings were made daily (3-6 hours per day), and chirps were recorded for 63 (52%) of the 122 adult males. The recorded audio files were divided into chunks (usually 5-10 seconds) and processed for later analysis. For this study, we used the calling songs of males during their first 30 days as adults, bringing the total number of recorded songs to 300 songs. The number of recordings at each age (including recordings of males more than 30 days after becoming adults) is shown in Figure 1C.

### 2.3. Computing environment

Songs were analyzed on a Macbook Pro (Retina, 15-inch, Mid 2015, OS X ver. 10.12.6, Apple Inc., Cupertino, California, USA) with R ver. 3.3.2, the R packages 'seewave' (ver. 2.0.5) and 'tuneR' (ver. 1.3.2), and Audacity version 2.1.1 (sound analysis software for Mac OS X; *http://audacityteam.org/*) was also used in this study.

### 2.4. Calculation of peak frequencies of calling songs

The recorded wave files were loaded into R using the 'seewave' package and a band-pass frequency filter (low-frequency cut-off 0.5 kHz, high-frequency cut-off 20 kHz) was applied. To obtain the peak frequency of the calling songs, the spectrum of each call was analyzed using the "spec" function of the package, which returned a value for the frequency and a corresponding value for the amplitude. The Nadaraya-Watson kernel regression estimation method was used to smooth the obtained data, and the frequency with the highest amplitude was taken as the peak frequency. This method yielded peak values comparable to those obtained with Audacity (using 'Plot Spectrum' function, AM pers. obs.).

2.5. Figure drawing

We used R ver. 3.3.2 to draw figures shown in this paper. Details are provided in the figure captions. The moving median calculation was applied to some of the longitudinal data for smoothing. Moving medians (or moving averages) are commonly used to visualize general trends in fluctuating time series data, but for the sake of data transparency, we also included unsmoothed data in these figures.

2.6. Helium-substitution experiment

A mixture of 80% helium and 20% oxygen (Manyusha, Tokyo, Japan) was used. First, crickets were placed in a plastic bag filled with room air (1 cricket/bag) and their chirps were recorded. The crickets were then transferred to a plastic bag containing the helium-oxygen mixture (1 cricket/bag) and their chirps were recorded. In this study, we used crickets that were 5 days old (post-adult age).

## 3. Results

3.1. Post-adult life history of *G. bimaculatus* used in this study

The maximum lifespan of *G. bimaculatus* used in this study (days counted from the last molt date) was 68 days, but more than 60% of the crickets died within 30 days (Figure 1A). Forewing coloring (lacquering) occurred very early after the final molt, with forewings appearing for the first time (tender) on the day of the final molt and gradually lacquering out after 3 days (Figure 1B). The number of records (reflecting both chirping frequency and survival of male crickets) peaked at day 4 (Figure 1C), and after normalization for survival, peaked between days 10 and 20 (Figure 1D). After the peak, the number of records (both absolute and normalized) was overall decreased toward the latter half of the adult stage (Figures 1C and 1D). This observation suggests that male sexual activity peaks around the second or third week of adult life. On the basis of these results, we decided to track changes in vocalizations within 30 days of the last molt. The following analysis covers 300 calling songs recorded from 63 adult males.

3.2. Maturation of calling song

In this study, crickets began to chirp on day 2 (1 recording was obtained on day 2, 8 on day 3, 22 on day 4, and 17 on day 5 (Figure 1C)). The frequency peaks were lower in the early adult stage than in the later adult stage (Figure 2). The median peak frequency of the calling songs was around 4.9 kHz on day 3, and reached 5.8 kHz on day 17 (Figure 2). Also, the frequency varied minimally around week 3 (Figure 2). After day 22, there was a slight downward trend in peak frequency and an increasing trend in the variability (Figure 2). We further tracked a representative individual over time, finding a shift in the peak frequency between days 5 and 8 for the individual. The representative male shown in Figure 3 chirped with a peak below 5.0 kHz on days 4 and 5,

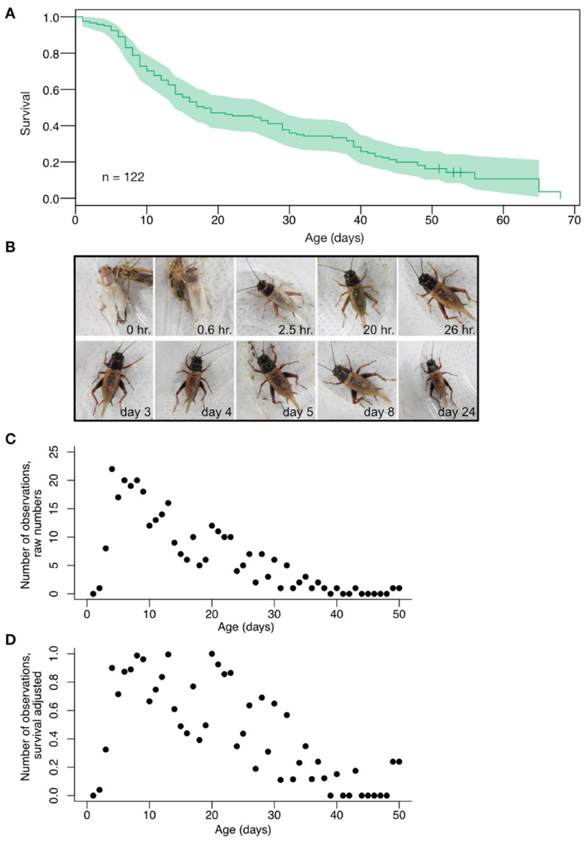


**Figure 1. Survival and wing development of the crickets used in this study. (A)** Survival curve of the crickets (*G. bimaculatus*) used in this study. The horizontal axis shows male post-adult age (counted from day of final molt), and the vertical axis shows survival. The green solid line shows the Kaplan-Meier curve of the crickets. Ticks indicate that one or more of the crickets were censored at that time-point (due to termination of experiment, or escaping). Green hatched area indicates 95% confidence intervals of the survival curve. This figure represents pooled survival data of 3 independent batches of crickets ($n$ = 122). Survival curves separately drawn for each batch are shown in Supplementary Figure S1. **(B)** Wing hardening of a male cricket after its final molt. Photos of a representative male *G. bimaculatus* were obtained over time. The time at the lower right corner of each photo indicates the interval between the final molt and the time at which the photo was obtained. The peak frequency of the calling song of this male was 4.9 kHz on day 4, and 5.3 kHz on day 24. **(C)** Raw numbers of successful recordings are plotted in the chart. The x-axis represents age (days), and the y-axis represents the number of recordings at each age. **(D)** The number of recordings adjusted by survival is shown in the chart for each age. Because the raw recording numbers represent both singing activity and survival on each day, we used the survival information (shown in the Figure S1) to normalize the data to demonstrate the singing activity per cricket at each age. The maximum activity was set to be 1 (y-axis). The x-axis represents the age (days).

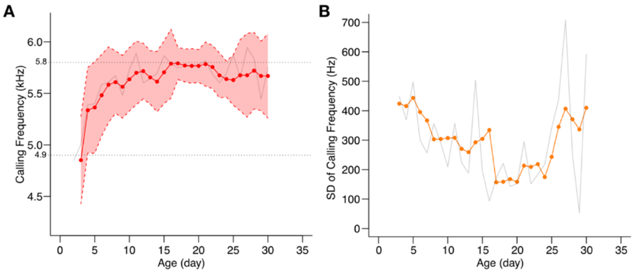


**Figure 2. Change in peak frequency of calling songs observed at the population level. (A)** Time-series data of the peak frequency of cricket calling songs. This chart represents 300 recorded calling songs from 63 males. The vertical axis shows peak frequency value of calling songs (kHz), and the horizontal axis shows cricket post-adult age (day of final molt = day 1). Red plots (connected by solid red lines) represent 3-day moving medians. The red-hatched region with red dashed lines indicates 3-day moving standard deviations. The number of samples (*i.e.*, recordings) obtained each day is shown in Figure 1C. The grey lines behind the red solid lines represent unsmoothed (*i.e.*, original time-series data of median peak frequency of calling songs. **(B)** Plot of standard deviation of peak frequency. The vertical axis indicates standard deviation of the peak frequency value (Hz), and the horizontal axis indicates the post-adult age of crickets. The red plots connected by red solid lines represent 3-day moving medians. The original, unsmoothed data are also shown in the chart with grey lines. The number of samples (*i.e.*, recordings) obtained each day is shown in Figure 1C.

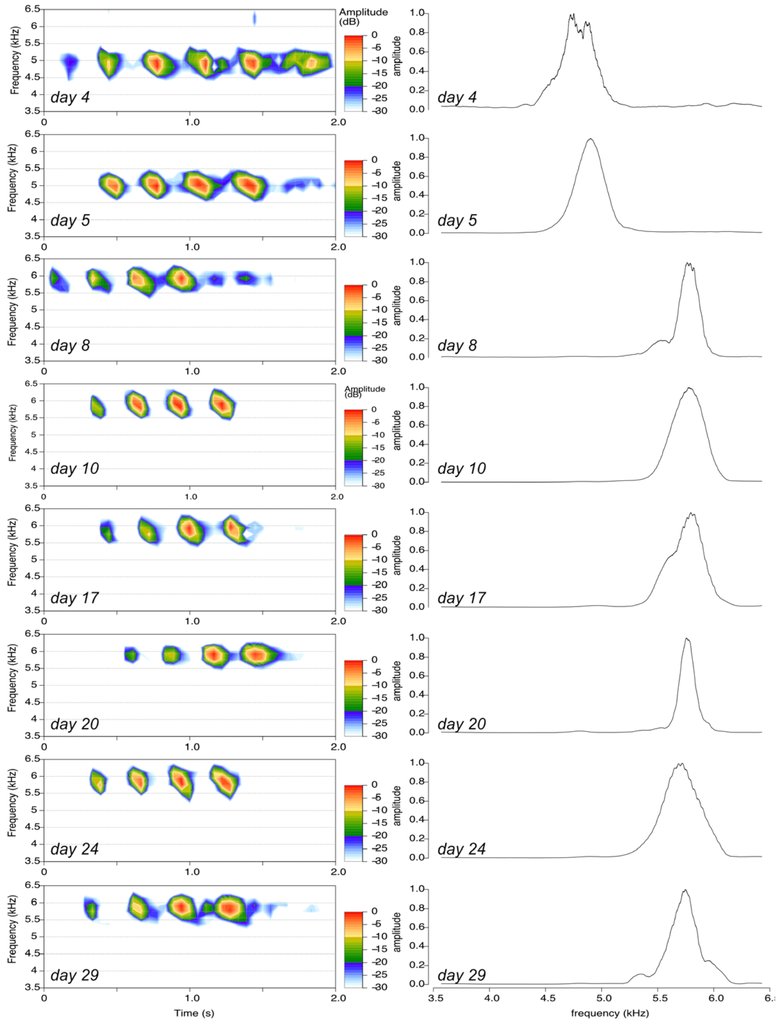


**Figure 3. Change in peak frequency of calling songs of a representative male.** Left panels are spectrograms of calling songs from a representative male that were recorded on different days. A chirp in each song is shown in the figure. The horizontal axis indicates time (seconds), and the vertical axis indicates frequency value (kHz). Color indicates relative amplitude of the frequency component as shown in scales on the right side of each chart. Figures were drawn by the 'spectro' function of the Seewave package running on R. The male ages for each chart are indicated at the lower left corner of each chart. Right panels demonstrate the distribution of the frequency components. The horizontal axis shows frequency values (kHz), and the vertical axis indicates the relative amplitude of each component. The amplitude values were normalized such that the maximum values = 1.0. The date at the lower left corner on each chart indicates male age. The distribution was calculated from a set of calling songs on each day (typically 10 seconds of recorded calling songs), using the 'spec' function of the Seewave package. Data were then smoothed for presentation purposes.

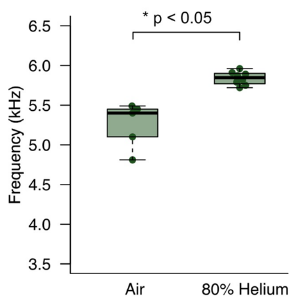


**Figure 4. Change in peak frequency of calling songs of young males in a helium-substituted environment.** The vertical axis shows the peak frequency value of the calling song. The labels below the horizontal axis indicate the group: 'Air' represents the calling songs recorded in normal room air conditions, and '80% Helium' represents calling songs recorded the helium-substituted condition. Males on day 5 were used for this experiment. Data from multiple different males were pooled (not all males emitted calling songs in both conditions, as sexual activity is low in early adult phase), and used for the analysis. Five samples were obtained for the normal condition and 8 were obtained for the helium-substituted condition. The peak frequency differed significantly between the 2 groups ($p < 0.05$ in Wilcoxon rank sum test).

and a peak around 5.8 kHz from day 8, a level that was maintained thereafter (Figure 3).

### 3.3. Effect of physical resistance on the peak frequency

The above finding that immature songs contain a low frequency peak indicates that the forewing membrane vibrates more slowly in immature males. We hypothesized that this was because some physical factor was preventing proper movement of the forewings. If this hypothesis is correct, then modifying the physical parameters that affect forewing movement should modify the peak frequency value of the chirp to an appropriate value. Thus, we tested whether crickets produce chirps with the proper pitch (peak frequency of 5.8 kHz) by reducing the physical resistance of the surrounding gas molecules. As shown in Figure 4, the chirps of young crickets (day 5) recorded in a helium-substituted environment (80% helium, 20% oxygen, average molecular weight (ignoring water content) = 9.6) were significantly higher than those recorded under normal air (Figure 4). The average frequency value of the songs recorded under helium replacement conditions was 5.8 kHz (Figure 4), which is comparable to that of the mature calling songs (see Figure 2A). This finding supports the idea that physical resistance from surrounding gas molecules affects the pitch regulation of cricket chirps, resulting in lower and more variable frequency peaks in the early adult male chirps.

## 4. Discussion

The present study demonstrated the development of song pitch in male calling songs during the post-adult maturation phase of *G. bimaculatus*, which reveals developmental aspects of our previous report that the frequency value of calling songs is robustly regulated during the late adult (7-14 days after final molt) phase (*9*). Female ears may be tuned to a certain pitch in orthopterans (*7,18-20*), suggesting that songs with improper pitches are ignored (or cannot be located) by females and thus have little reproductive contribution. Why then do immature males bother to sing if their songs will not be located? A possible explanation is that they learn to sing by producing sounds at an early stage (*e.g.*, it may require feedback from their auditory system). Crickets are capable of modifying their song frequencies by auditory feedback systems (*24*). From this perspective, an interesting question to address in future studies is whether interfering with the learning process (*e.g.*, damaging their auditory organs) disrupts song development.

Another explanation from an ethologic aspect is that songs from immature males may act as a decoy to predators. One of the reproductive costs of cricket songs is that they increase the predation risk (*25*). If the songs attract predators and only sexually mature males sing (and immature males keep silent), the predation risk should be biased toward older and sexually mature males. Being older means that they have survived several life-threatening risks, such as infection. Therefore, it seems advantageous that young, sexually immature males would sing even though their songs do not attract conspecific females, as they can act as decoys to protect more fit and sexually mature males from predators.

What molecular and physiologic/biophysical mechanisms explain song development? The sound producing mechanism in crickets demonstrated in previous studies (*8,24*) suggests that muscle development, tegmina condition (*e.g.*, moisture content or tension), developmental state of the neural circuit, and other factors affect cricket song frequency. In a simple biophysical model, the parameters that determine the vibration frequency of membrane instruments are density, tension, and size (diameter). Also, the shape of the membrane and how the initial vibration is given could affect the final vibration pattern. In the song development demonstrated in this study, a decrease in moisture content (*i.e.*, sclerotization) of the forewings may help to stabilize vibration and explains the robust song pitch at 5.8 kHz. The helium substitution experiment in this study demonstrates that physical resistance from surrounding gas molecules negatively affects the stabilization of song pitch. As a future study, vibratory properties of forewings could be observed using a laser Doppler vibrometer either in normal air or in a helium-substituted condition to further confirm this speculation.

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*Conflict of Interest*: The authors have no conflicts of interest to disclose.

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